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The *Pseudomonas syringae* type III effector HopE1 interacts and inhibits an  
*Arabidopsis* WNK Kinase

A Thesis submitted in partial satisfaction of the requirements  
for the degree Master of Science

in

Biology

by

James L. Engel Jr.

Committee in charge:

Professor Jack Dixon, Chair  
Professor Steve Briggs, Co-Chair  
Professor Nigel Crawford

2008

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Co-Chair

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Chair

University of California, San Diego

2008



## DEDICATION

I would like to dedicate this thesis to my family.  
Thank you Mom and Dad for always being there and helping me along the way. Thanks to my sister April, who has entertained and watched out for me along the way and to my niece Mariyah and nephew Carson.

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## ABSTRACT OF THE THESIS

The *Pseudomonas syringae* type III effector HopE1 interacts and inhibits an *Arabidopsis* WNK Kinase

by

James L. Engel Jr.

Master of Science in Biology

University of California, San Diego, 2008

Professor Jack Dixon, Chair  
Professor Steve Briggs, Co-Chair

Bacterial phytopathogens employ a type III secretion system to deliver effector proteins into the plant cell to suppress defense pathways, however the underlying molecular mechanisms of these effectors largely remain a mystery. Here, we demonstrate that the *Pseudomonas syringae* pv. *tomato* (*Pst*) type III effector, HopE1, specifically binds *Arabidopsis* With No Lysine (K) protein kinase 8 (WNK8) using a yeast two-

hybrid screen, purified recombinant proteins, wheat germ *in vitro* transcribed and translated proteins, as well as *Arabidopsis* cell extracts. We also clearly show that WNK8 phosphorylates HopE1 and that HopE1 inhibits the autophosphorylation of WNK8 as well as the WNK8 phosphorylation of subunit C of the *Arabidopsis thaliana* vacuolar H<sup>+</sup>-ATPase (AtVHA-C). Furthermore, we demonstrate that *WNK8* expression is induced upon *Pst* infection suggesting a possible role in early defense response. HopE1 and other effectors have been shown to suppress the plant hypersensitive response in order to promote pathogen survival. Although the precise molecular mechanism or *in planta* substrates of HopE1 remain obscure, our work uncovers a potential substrate and creates the tools necessary to further examine the role HopE1 plays in plant pathogenesis.

## Introduction

Every year, millions of valuable crops are lost to disease and is estimated to cost producers billions of dollars in revenue worldwide (Agrios, 2005). As the human population continues to expand, the importance of food availability and dependence on crops becomes an even greater issue. In order to feed the world's population the issue of crop disease must be addressed in the very near future. Many of the crops lost are due to various phytopathogens that are evolutionary tailored to infect specific plant species that elicit a variety of disease progressions including bacterial canker, bacterial spot, bacterial speck, tomato-tobacco mosaic virus, and fungal leaf spot diseases.

Bacterial speck disease was a major cause of tomato crop loss and quickly became an obstacle in tomato production until the *Pseudomonas* tomato (Pto) resistance gene was introduced in the 1970's. Bacterial speck disease in tomatoes is caused by many strains of the Gram-negative bacterial phytopathogen *Pseudomonas syringae*. The pathogenicity of one particular strain, *Pseudomonas syringae* pv. *tomato* (*Pst*) is dependent on the ability to inject virulence factors (effector proteins) into the host cell via a type III secretion system (TTSS) encoded by hypersensitive response and pathogenicity (*hrp*) and hrp conserved (*hrc*) genes (Alfano and Collmer, 2001). For example, the *Pst* DC3000 strain has been shown to secrete approximately 30 different proteins into the host plant cell via a TTSS that define host specificity and as a collection, carry out the virulence of this pathogen (Chang et al., 2005; Petnicki-Ocwieja et al., 2002; Roine et al., 1997). Although some of these secreted proteins have been identified and characterized, the function of many effectors remain largely unknown and unstudied.

Phytopathogen research is focused on two important facets of the plant-pathogen interaction: 1) the strategies employed by bacterial effectors to cause a particular disease response in the host and 2) the molecular mechanisms of host defense responses launched by the plant to eliminate the pathogen. Once secreted into the plant cell via the TTSS, the bacterial effectors carry out two types of infections. In an avirulent infection, avirulence (Avr) genes encode bacterial Avr proteins that elicit localized programmed cell death or the hypersensitive response (HR) in the host. This potent response creates an incompatible plant-pathogen interaction since it often occurs with cessation of pathogen growth (Scheel, 1998) in a resistant host. In a compatible infection, virulence genes enable bacterial expansion and subversion of host defense responses resulting in disease progression of a susceptible host (Ausubel et al., 1995).

The study of plant defense responses and the signaling pathways targeted by pathogens have come to the forefront of phytopathogen research. The ultimate goal will be to capitalize on these findings to engineer pathogen resistant plants and crops. Such research could also reduce the overuse and abuse of harmful pesticides. Plants have evolved a two-tier type of immunity to pathogens that include both basal and specific defense responses. The primary or basal plant defense response is one of general surveillance that is dependent on the ability of the plant cell to recognize conserved microbe-associated molecular patterns (MAMPS) such as bacterial flagellin or lipopolysaccharide structures through a set of conserved cell surface receptors referred to as pattern recognition receptors (PRRs) (Nurnberger et al., 2004; Zipfel and Felix, 2005). Upon MAMP recognition, the plant launches a non-specific defense response that includes the release of anti-microbial growth agents such as chitinases, glucanases and



proteases as well as cell wall alterations to prevent microbe invasion (de Wit, 2007; van Loon et al., 2006). The more specific secondary defense mechanism involves a “gene for gene” resistance model. In this model, the pathogen injects Avr proteins into the plant cell via the TTSS and when a single plant resistance (R)-protein recognizes a single Avr protein, the plant launches a highly specific battery of defense responses intended to interfere with the multiplication and survival of the pathogen (Kim et al., 2008). In *Arabidopsis thaliana*-*P. syringae* incompatible interactions, R-proteins recognize effector proteins by various mechanisms and induce signaling cascades in their respective pathway. These pathways ultimately lead to defense mechanisms such as rapid ion fluxes, intracellular and extracellular oxidative bursts, changes in phosphorylation status, and induction of salicylic acid (SA) responses (Holt et al., 2000; Shirasu and Schulze-Lefert, 2003). These defense responses play an extremely vital role in disease resistance, restriction of pathogen growth and induction of HR.

The plant hypersensitive response has been characterized as a localized programmed cell death (PCD) reaction at the site of infection (Alfano and Collmer, 1996), which often occurs concomitantly with suppression of pathogen growth (Scheel, 1998). Induction of HR is an important defense response that plants use to thwart invading bacteria. Recent studies suggest that some phytopathogens, notably *Pst* DC3000, have maintained and tailored a set of TTSS effectors to suppress HR by targeting host HR defense pathways (Petnicki-Ocwieja et al., 2002). Jamir and colleagues recently examined TTSS effectors that are able to suppress PCD or HR using two different suppression assays (Jamir et al., 2004). The first of these experiments test for the ability of certain effectors to suppress PCD induced by the mammalian pro-apoptotic

protein Bax in yeast (*Saccharomyces cerevisiae*) and tobacco (*Nicotiana benthamiana*) when co-expressed in the organism. These experiments have identified effectors that suppress Bax-mediated cell death, however, this system is highly artificial. A functional equivalent of Bax has yet to be identified in any plant species (Xu and Reed, 1998), but Bax inhibitor homologues have been identified in *Arabidopsis* (Sanchez et al., 2000). The mechanism by which Bax induces PCD *in planta* remains unknown. In a second set of experiments, Jamir and colleagues induce HR in tobacco, *N. tabacum* cv. *Xanthi*, and *A. thaliana* Ws-0 using the pHIR11 cosmid in order to further study their set of effectors for the suppression of HR. The pHIR11 cosmid allows non-pathogens such as *P. fluorescens* to elicit HR in a TTSS dependent manner because it carries a Hrp pathogenicity island from *P. syringae* pv. *syringae* 61. The Hrp pathogenicity island encodes a functional TTSS and the avirulence protein HopPsyA. HopPsyA is recognized by R-protein(s) in tobacco and *Arabidopsis* Ws-0 causing HR (Alfano and Collmer, 1997; Huang et al., 1988; van Dijk et al., 2002). Interestingly, of the 19 effectors tested only 2 of these effectors, HopE1 (formerly known as HopPtoE) and HopF2 (formerly known as HopPtoF), suppress both Bax induced PCD and HopPsyA induced HR, while 5 others only inhibit one of these HR reactions. The large number of effectors able to suppress the plant HR indicate HR suppression is vital for *P. syringae* pathogenesis. Therefore, we chose to study the *Pst* effector HopE1 in more detail in order to elucidate its role in pathogenesis and HR suppression.

We choose to investigate the potential molecular targets of HopE1 during pathogenesis. We used the yeast two-hybrid system to identify potential *Arabidopsis* proteins that are able to interact with HopE1 followed by extensive biochemical

experiments to substantiate these interactions. We propose that the *Pseudomonas syringae* pv. *tomato* DC3000 type III effector HopE1 targets the *Arabidopsis* with no lysine (K) (WNK) kinase-8 (WNK8) during infection. Using the yeast two-hybrid system, purified proteins, *in vitro* transcribed and translated proteins, as well as *Arabidopsis* cell extracts we demonstrate that HopE1 interacts with WNK8 *in vitro*. Knowing that HopE1 and WNK8 bind, we then investigated whether WNK8 can phosphorylate HopE1 *in vitro*. We found that WNK8 phosphorylates HopE1 and more interestingly we show HopE1 can alter the activity of WNK8 by inhibiting WNK8 autophosphorylation. We also use reverse transcription polymerase chain reaction (RT-PCR) to show that WNK8 is upregulated during *Pst* infection providing further evidence that WNK8 may be involved in the defense response signaling or HR during bacterial pathogenesis and is a potential target of HopE1 *in planta*.

WNK8 is a member of the STE20/PAK subfamily of serine/threonine WNK protein kinases (Xu et al., 2002) whose closest homologues are part of the mitogen-activated protein kinase (MAPK) signaling pathway (Cope et al., 2005). These kinases are so-named due to the otherwise conserved catalytic lysine residue normally found in sub-domain II of the traditional protein kinase domain is actually found in sub-domain I of these kinases. Although WNK kinases contain this change in sub-domain organization, based on the solved crystal structure of rat WNK2, the three-dimensional configuration of the active site cleft remains unchanged (Huang et al., 2007). The *Arabidopsis* family of WNK kinases contain 11 members whereas the human WNK family contains only 4 members. All members of the human WNK family have been implicated in hypertension and responsible for the regulation of ion transport and regulation mostly in the renal

system (Gamba, 2005), there is currently no evidence that a similar function is carried out in plant species despite having significantly more family members. *Arabidopsis thaliana* WNK8 has been proposed to be involved in pH regulation by binding to and phosphorylating subunit C of the *Arabidopsis* vacuolar H<sup>+</sup>-ATPase (V-ATPase) *in vitro* (Hong-Hermesdorf et al., 2006). Based on this finding and our data, it would be interesting to determine if HopE1 is indirectly misregulating the V-ATPase channel by disrupting WNK8 activity during infection and whether or not this has implications in defense signaling or HR.

## Results and Discussion

### *A Yeast Two-Hybrid Assay Identified Potential HopE1 Interacting Proteins*

In order to identify potential molecular targets of HopE1, a Gal4-based yeast two-hybrid screen was performed using full length HopE1 as the bait protein, which was screened against an *Arabidopsis* cDNA library in the L40 strain of *S. cerevisiae*. The L40 yeast strain contains an integrated yeast *HIS3* reporter gene under the control of four LexA operators. Upon interaction between the Gal4-binding domain and the LexA-activation domain transcription of the *HIS3* reporter is initiated and positives can be selected by growth on minimal synthetic media (SD) lacking histidine (selection for *HIS3* reporter), tryptophan (selection for Gal4 library prey plasmid) and uracil (selection for bait plasmid) (Vojtek et al., 1993). To eliminate the possibility of false positives due to HopE1 autoactivation of the *HIS3* reporter, the bait plasmid was tested in the absence of the library plasmid. This resulted in no growth on SD-Ura-His agar plates (data not shown) suggesting a very low false positive rate for our screen. To verify the expression of the LexA-HopE1 bait protein, yeast lysates were analyzed using an anti-LexA antibody (Fig. 1A). After testing for auto-activation and bait expression, the yeast two-hybrid assay was carried out using a cDNA library generated from 3 day old etiolated *Arabidopsis* as previously described (Kim et al., 1997) and a total of 35 million colonies were screened. After selection on SD-Trp-Ura-His minimal media, positives were then grown in SD media lacking only tryptophan to drop out the bait and select for yeast

containing only the library plasmid, which was then isolated, transformed into *E. coli* and sequenced until saturation of clones was reached. The resulting nucleotide sequences were then compared to the *Arabidopsis thaliana* genome using the BLASTN algorithm (Altschul et al., 1990) to identify the yeast two-hybrid positive clones (Fig. 1B). These results gave us a limited subset of proteins that potentially interact with HopE1, which were narrowed down further to include those proteins that may have functions involved in plant disease response or defense signaling pathways based our intuition. We ultimately decided to focus on two interacting proteins, calmodulin 7 (CaM7) and WNK8.

We performed *in vitro* binding assays using purified proteins to test for a biochemical interaction with HopE1. No interaction was detected with CaM7 (data not shown), but a positive interaction was detected with WNK8, therefore we focused on further characterizing the HopE1/WNK8 interaction. We performed a forced yeast-two hybrid with the full length WNK8 protein identified in our initial screen to test the specificity of the interaction. L40 yeast was co-transformed with *WNK8* or *WNK10* preys and *HopE1* bait plasmids and three independent isolates were assayed on both SD-Trp-Ura minimal media to test for yeast viability and SD-Trp-Ura-His minimal media to test for a positive interaction (Fig. 1C). We found that WNK8-wild-type (WT) tightly associated with HopE1 in the forced yeast two-hybrid. To determine if an active kinase is required for the interaction, we tested WNK8-K41M (kinase dead) and found that it interacts equally as well as WT. We also examined the specificity of the HopE1/WNK8 yeast interaction by testing the most similar *Arabidopsis* WNK kinase, WNK10 (Hong-Hermesdorf et al., 2006). Our data indicates that an active kinase is not required for the

interaction since both WNK8-WT and WNK8-K41M preys show growth on SD-Trp-Ura-His minimal media. Additionally, the interaction is specific to WNK8 since WNK10 prey growth is equal to that of background (pGADGH EV). We also determined that these preys do not auto-activate since no growth was seen when grown on SD-Trp-Ura-His minimal media in the absence of the bait plasmid. These data indicate that the HopE1/WNK8 yeast two-hybrid interactions do not require kinase active WNK8 and are specific since no HopE1/WNK10 interaction was detected in both our initial screen as well as our forced yeast two-hybrid screen.

#### *Purified HopE1 and WNK8 Interact*

In order to substantiate our yeast two-hybrid results, we tested the ability of HopE1 and WNK8 to form a complex in a purified state. To this end we expressed and purified recombinant N-terminally Glutathione S-transferase (GST) tagged wild-type HopE1 (WT) and C-terminally His<sub>6</sub> tagged WNK8-WT in *E. coli*. Additionally we purified the known kinase dead mutants, WNK8-K41M and WNK8-D157A (Hong-Hermesdorf et al., 2006), as well as WNK10 (maltose binding protein-WNK10-His<sub>6</sub>). Recombinant GST, GST-HopE1 or GST-AvrPtoB 309-55 (negative control) proteins were then incubated with increasing amounts of WNK8-His<sub>6</sub> WT, WNK8-His<sub>6</sub> K41M, WNK8-His<sub>6</sub> D157A, MBP-WNK10-His<sub>6</sub> WT or a mixture of these proteins. After incubation, we performed a GST pull down and analyzed the interaction by SDS-PAGE and western blotting with an anti-His<sub>4</sub> antibody. Binding was detected in all samples containing GST-HopE1 and WNK8-His<sub>6</sub> WT, K41M or D157A, but not in samples

containing GST, GST-AvrPtoB 309-553 (Fig. 2), demonstrating that the interaction is not between the GST affinity tag and WNK8, but between HopE1 and all kinase forms of WNK8. Although MBP-WNK10-His<sub>6</sub> weakly interacts with HopE1, this interaction is effectively competed off by the addition of WNK8-His<sub>6</sub> WT protein. This indicates that HopE1 preferentially interacts with WNK8 over WNK10. Additionally, we show that WNK8 preferentially interacts with HopE1 because the addition of AvrPtoB 309-553 had no effect on the HopE1/WNK8 interaction. These data indicate a strong and specific interaction between purified HopE1 and WNK8 that is independent of WNK8 kinase activity.

#### *WNK Kinase Architecture and Sequence Analysis of WNK8 and WNK10*

The *Arabidopsis* WNK family of kinases have similar domain architectures that include a N-terminal WNK kinase domain (KD), an autoinhibitory domain (AID) and a predicted C-terminal coiled-coil (CC) domain (Fig. 3A). Since HopE1 bound strongly to WNK8, but weakly to WNK10, we analyzed the domain structure of the two proteins with regard to the differences in the amino acid sequence of these two proteins. We aligned the amino acid sequences of WNK8 and WNK10 using ClustalW (Fig. 3B) and compared specific regions throughout the proteins for the percentage of identical residues and the percentage of similar (chemically conserved) residues (Fig. 3C). Based on this, we found that the full-length WNK8 and WNK10 proteins are highly similar (77%), with the highest level of conservation observed in the KD (94%) and in the CC domain (91%) while the AID's were more divergent (76% similar). Therefore the weak HopE1 binding



to WNK10 that we observed is probably due to these conserved regions among all WNK kinases. In order to obtain tight binding as observed with WNK8, binding presumably occurs outside of these regions as well. For example, the region between the KD and the AID (291-382) is the region of least similarity (68%) and the region between the AID and the CC domain (422-516) also has lower similarity (73%) than the conserved WNK domains and contains an interesting 16 amino acid insertion in the WNK8 sequence. The differences observed between the primary sequences of WNK8 and WNK10 suggest that the regions of lower similarity may play a key role in the substrate recognition and binding specificity of WNK8.

#### *In vitro Transcribed and Translated WNK8 Interacts with HopE1*

To further examine the HopE1/WNK8 binding interaction, we tested the interaction at reduced concentration using *in vitro* transcribed and translated (TnT) proteins. Purified GST-HopE1 and control proteins were immobilized to glutathione beads before addition of TnT generated <sup>35</sup>S-methionine labeled WNK8-His<sub>6</sub> or WNK10-His<sub>6</sub> proteins produced in wheat germ extract. We then performed GST pull downs and analyzed the HopE1/WNK8 interaction by SDS-PAGE and <sup>35</sup>S-autoradiography. Significant binding was detected between HopE1 and WNK8-WT as compared to GST background binding (Fig. 4). HopE1 binding was also detected with the WNK8-K41M and less with WNK8-D57A while weak binding was detected for WNK10. Equal amounts of proteins were added to each reaction as indicated by the coomassie stained membrane (Fig 4). The small amount of WNK10 binding we detected is probably due to

the high similarity between WNK8 and WNK10 in certain regions, but HopE1 clearly binds stronger WNK8 than WNK10. These data strengthen the case for a specific interaction between HopE1 and WNK8 because of the low concentrations of WNK8 that are produced in the TnT reaction as well as the complex mixture of TnT and wheat germ proteins added to the GST pull down.

#### *Validating WNK8 T-DNA Insertion Lines*

In order to further examine the role WNK8 plays in defense response, we obtained two different *Arabidopsis* Col-0 Salk T-DNA insertion lines. The SALK\_103318 (designated *wnk8-1*) and SALK\_024887 (designated *wnk8-2*) lines both contain an insertion in a *WNK8* exon (Alonso et al., 2003). We initially isolated homozygous insertion mutants and analyzed them for disruption of the *WNK8* gene. We isolated genomic DNA from 24 individual *wnk8-1* plants and 24 *wnk8-2* plants and tested them for the insertion using PCR analysis (Fig. 5A). We isolated several homozygous mutant plants verified by PCR based genotyping. The T<sub>2</sub> plants were tested using real-time quantitative PCR (RT-qPCR) analysis to verify disruption of the *WNK8* transcript (Fig. 5B). Based on these experiments, we conclude that the isolated homozygous seed from the *wnk8-1* line is not a suitable insertion line since *WNK8* expression is elevated (386% of Col-0) as normalized to WT Col-0 (100%) plants, however the *wnk8-2* insertion line is a functional knockout of *WNK8* transcript (7% of Col-0). We used this insertion line in all subsequent experiments.

### *HopE1 Interacts with WNK8 in Arabidopsis Cell Extracts*

In order to supplement our *in vitro* interaction data, we tested the HopE1/WNK8 interaction under near physiological conditions that more closely represent the conditions during infection. Because there are no WNK8 antibodies available, we expressed epitope tagged versions of both proteins *in planta* and were able to immunoprecipitate (IP) these proteins from *wnk8-2* plants. C-terminal hemagglutinin (HA) tagged HopE1 was over-expressed using a dexamethasone inducible 35S promoter in *wnk8-2* plants. HopE1 was co-immunoprecipitated from Dex inducible *wnk8-2* 35S::*hopE1-HA* plant whole cell lysate (WCL) when incubated with recombinant WNK8-His<sub>6</sub> protein bound to Ni-NTA beads (Fig. 6A). We were also able to co-immunoprecipitate HopE1 from Dex inducible *wnk8-2* 35S::*hopE1-HA* plant whole cell lysate (WCL) when incubated with Flag-WNK8 immunoprecipitated from *wnk8-2:Flag-WNK8* plant WCL using Flag M2 agarose bead (Fig. 6B). The N-terminal Flag tagged *WNK8* was expressed at endogenous levels by complementing Flag-*WNK8* driven by its native promoter into the *wnk8-2* null insertion line. These data illustrate that HopE1 and WNK8 can interact under conditions relevant to infection and further substantiates our previous *in vitro* binding data. We have shown that the HopE1/WNK8 interaction occurs in a specific manner using multiple biochemical approaches and we would therefore hypothesize that this interaction occurs *in planta* as well.

### *WNK8 Expression Levels During Pseudomonas syringae Infection*

To investigate if WNK8 plays a role during pathogen infection, we tested the expression level of WNK8 in *Arabidopsis* Col-0 plants during a high density *Pst* DC3000 infection using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Infected leaf samples were collected at various time points, the RNA was isolated, converted to cDNA via RT and amplified by PCR using *WNK8* or actin (internal PCR control) specific primers to analyze the *WNK8* transcript levels (Fig. 7A). It is not currently known if WNK8 is involved in the *Arabidopsis* defense response, although it is likely that some genes involved in pathogen defense undergo rapid and prolonged transcriptional changes. Our data suggest a possible role for WNK8 in early defense response because the expression level is elevated within the first hour post infection, remains elevated for 6 hours following infection and decreases back to basal levels by 24 hours post infection (h.p.i). These data are further supported by publicly available *Arabidopsis* microarray data (Craigon et al., 2004), which also shows upregulation of *WNK8* transcript during different types of compatible and incompatible infections (Fig. 7B). Consistent with our results, the microarray data shows increased *WNK8* transcript early in infection (2 h.p.i). However this transcript remains elevated for at least 24 hours post infection. It is important to note that the microarray data was produced from *Arabidopsis* leaf tissue that was infected at low density while our RT-PCR data was generated using a high density *Pst* infection, which can lead to differences in disease progression and thus differences in gene transcript level. However, these two independent experiments clearly suggest that WNK8 may be involved in the *Arabidopsis* early

defense response and implicate WNK8 as a possible molecular target for effector proteins during pathogen infection.

### *WNK8 Phosphorylates HopE1 in vitro*

Given that WNK8 and HopE1 associate and that WNK8 is an active protein kinase (Hong-Hermesdorf et al., 2006), we next tested if WNK8 phosphorylates HopE1 using an *in vitro* kinase assay. In this assay, we incubated purified WNK8-His<sub>6</sub> with either GST-HopE1 or MBP-HopE1 in the presence of radiolabeled  $\gamma$ -<sup>32</sup>P adenosine triphosphate (ATP) and necessary kinase co-factors. Our data demonstrate that WNK8 autophosphorylates and can phosphorylate HopE1 *in vitro* (Fig. 8A). Although GST-HopE1 is clearly phosphorylated in the presence of WNK8-WT, the kinase dead WNK8-D157A mutant or WNK10-WT did not phosphorylate HopE1 despite the use of equal molar amounts of the kinases (Fig. 6A). These data are intriguing since HopE1 weakly binds WNK10, but cannot be phosphorylated by WNK10 *in vitro*. To further substantiate the *in vitro* phosphorylation of HopE1, we expressed and purified thrombin cleavable MBP-HopE1 protein and performed kinase assays (Fig. 8B). Since phosphorylation of MBP is a known artifact of *in vitro* kinase assays (Asthagiri et al., 1999), we wanted to eliminate the possibility that the <sup>32</sup>P was being incorporated onto the MBP affinity tag and be able to detect phosphorylation on untagged full length HopE1. We performed a thrombin cleavage of MBP-HopE1 following the kinase assay to remove the tag (Fig. 7B). Following thrombin cleavage, HopE1 becomes unstable and precipitates out of solution, which was then be analyzed by <sup>32</sup>P autoradiography. We also detected WNK8

by autoradiography indicating that some WNK8 precipitates during the thrombin cleavage as well, but this has no effect on analyzing the  $^{32}\text{P}$  incorporation in HopE1. These data clearly show the rapid phosphyl-transfer to HopE1 (occurs in under 30 min) and not MBP. Together, our results indicate that not only is the HopE1/WNK8 interaction specific, but that the *Pst* effector protein HopE1 is selectively phosphorylated *in vitro* by the *Arabidopsis* WNK8 kinase. The implications of this phosphorylation event *in planta* remain unknown.

It is possible that WNK8 activates HopE1 by phosphorylation, which is required for the activity of HopE1 during pathogenesis. This important modification may be required to cause a conformation change in the structure of HopE1 converting it into an active protein. It has been previously shown that another *Pst* effector, AvrPtoB, undergoes this same modification and that it is required for activity (Xiao et al., 2007). It is unknown which kinase activates AvrPtoB *in planta*, but Xiao and colleagues have identified the phosphorylation site and have shown that when this site is mutated the virulence and avirulence activity of AvrPtoB is lost. It still remains unknown if HopE1 is phosphorylated by WNK8 *in planta*. Future efforts would include mapping the HopE1 phosphorylation site by mass spectrometry and performing similar *in planta* experiments to Xiao and colleagues to show the dependence of HopE1 phosphorylation *in planta*.

#### *HopE1 Alters the Kinase Activity of WNK8*

Based on our binding experiments, we hypothesized that HopE1 might alter the WNK8 kinase activity as a virulence function of HopE1 during pathogen infection. Using

the *in vitro* kinase assay, we tested if HopE1 could inhibit the autophosphorylation of WNK8. We added increasing molar amounts of HopE1 to a constant amount of WNK8 in order to detect a concentration-dependent inhibition of WNK8 autophosphorylation activity (Fig. 9A). Our data show that HopE1 suppresses WNK8 autophosphorylation activity in a concentration-dependent manner, indicating that HopE1 is negatively regulating the ability of WNK8 to autophosphorylate. We are confident that the inhibition is HopE1 dependent since adding an excess amount of GST had no effect on WNK8 autophosphorylation activity. To eliminate the possibility that the decrease in WNK8 autophosphorylation is a nonspecific phenomena due to binding and phosphorylating a substrate, we performed a similar assay using a known WNK8 substrate, AtVHA-C 135-267 (Hong-Hermesdorf et al., 2006). We observed no change in the autophosphorylation status of WNK8 (Fig. 9B). Next, we determined if the HopE1 induced suppression of WNK8 autophosphorylation inhibits phosphorylation of the AtVHA-C peptide substrate in the *in vitro* kinase assay. To test this, we added AtWNK8 and AtVHA-C 135-267 at equal molar concentrations, as well as increasing molar amounts of HopE1 protein in a single kinase assay and evaluated the phosphorylation of each protein by autoradiography (Fig. 9C).

Based on our data, we propose two potential mechanisms for how HopE1 inhibits the phosphorylation of AtVHA-C. First, it is possible that HopE1 binds to WNK8 in a manner that blocks the ability of WNK8 to autophosphorylate, therefore WNK8 can no longer phosphorylate AtVHA-C. It is also possible that HopE1 inhibits the phosphorylation of AtVHA-C by chemically modifying WNK8 in a manner that inhibits WNK8 autophosphorylation. Future endeavors would investigate this question by

determining the biochemical activity of HopE1 and mapping the HopE1/WNK8 binding to specific regions on both proteins. Binding information would be valuable because HopE1 could be inhibiting the WNK8 autophosphorylation by binding simply to the kinase domain of WNK8 or binding to the WNK8 regulatory autoinhibitory domain. More importantly, showing the inhibition of WNK8 *in planta* and the effect this may have on *Arabidopsis* VHA-C could possibly identify the role HopE1 plays during *Pst* infection.

#### *Creation of a Pst DC3000 HopE1 Knockout Strain*

To further examine the importance of HopE1 in the *Pst* repertoire of effectors, we generated a HopE1 knockout strain of *Pst* DC3000. To create the HopE1 knockout strain, we inserted a kanamycin resistance gene cassette within the coding sequence of *hopE1* in the *Pst* genome by homologous recombination. Recombinants were isolated by plating the bacteria on selective media and then were genotyped by PCR using primers directed against *hopE1* as well as 3 other known *Pst* genes (*hopC1*, *hopG1* and *hopN1*). Two isolates were successfully created that contain the insertion (PCR product of 1500 bp instead of 636 bp) in the *hopE1* sequence (Fig. 10). The creation of a *Pst* DC3000  $\Delta$ *hopE1* is a good tool for determining the role HopE1 plays during *Pst* infection.

Future efforts will complement this strain with an epitope tagged form of HopE1, which will be used to infect *wnk8-1::Flag-WNK8* plants. Co-IPs will be performed to assess HopE1/WNK8 binding *in planta*. We also could use this strain to compare the kinase activity of immunoprecipitated WNK8 in *Pst* WT versus *Pst*  $\Delta$ *hopE1* infected

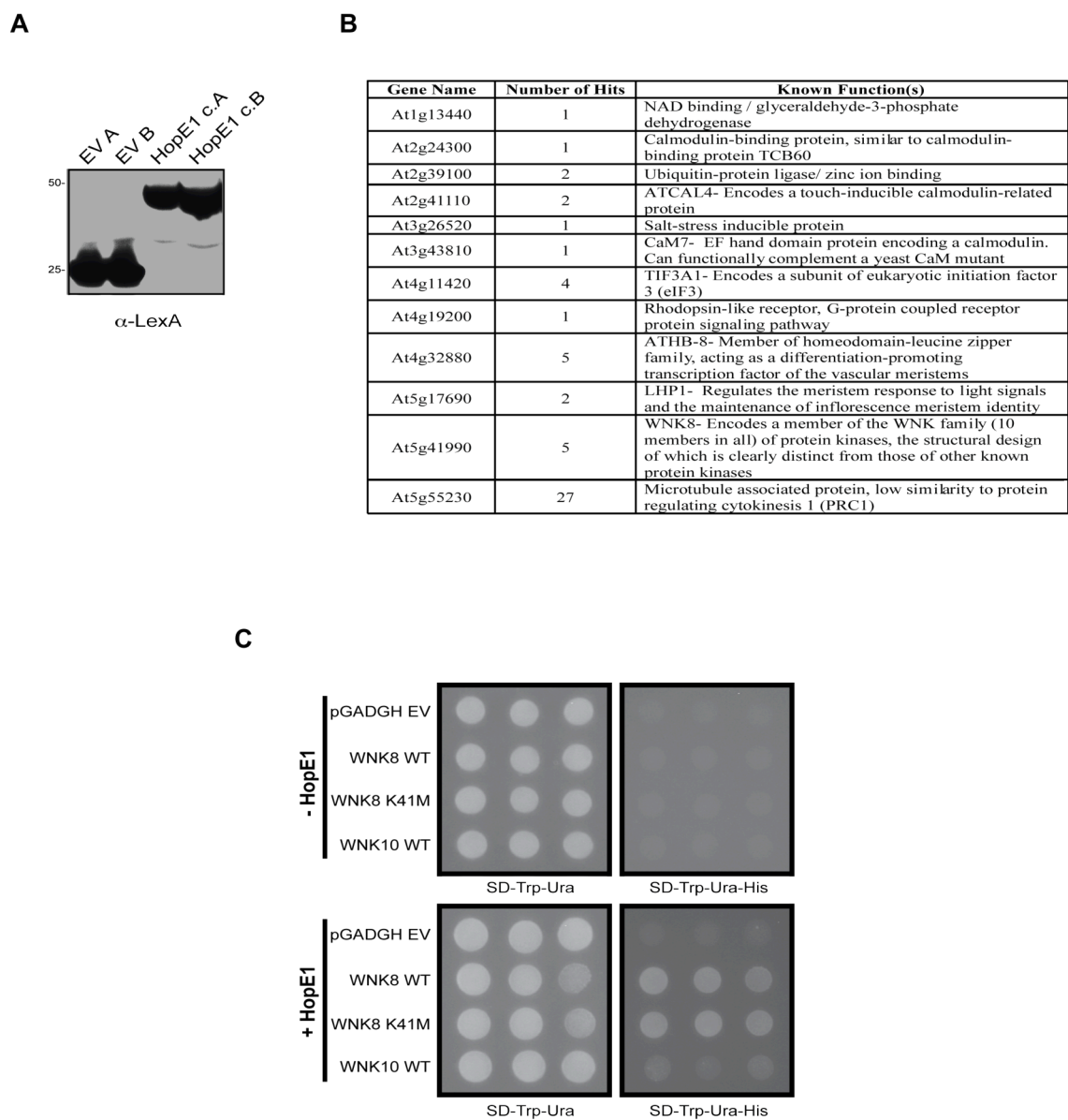


plants and determine if WNK8 is being modified in an irreversible manner. Furthermore, to substantiate if WNK8 is involved in the *Arabidopsis* HR we would perform ion leakage assays using Col-0 or *wnk8-2* plants infected with *Pst* WT and *Pst*  $\Delta$ *HopE1*. It is known that WNK8 regulates the *Arabidopsis* vacuolar H<sup>+</sup>-ATPase subunit C, which plays an important role in pH regulation (Hong-Hermesdorf et al., 2006). The WNK8 regulation of V-ATPase subunit C may play a role in HR by changing the cytosolic pH through the leakage of multiple ions controlled by these proteins. It has been shown that pH plays an important role in programmed cell death in *Arabidopsis* (Errakhi et al., 2008), which could implement VHA-C in this process as well. Since it has been shown that HopE1 can suppress HR (Jamir et al., 2004), the development of tools such as the *Pst*  $\Delta$ *hopE1* strains and *wnk8-2:Flag-WNK8* plants will be important in dissecting the function of HopE1 during infection.

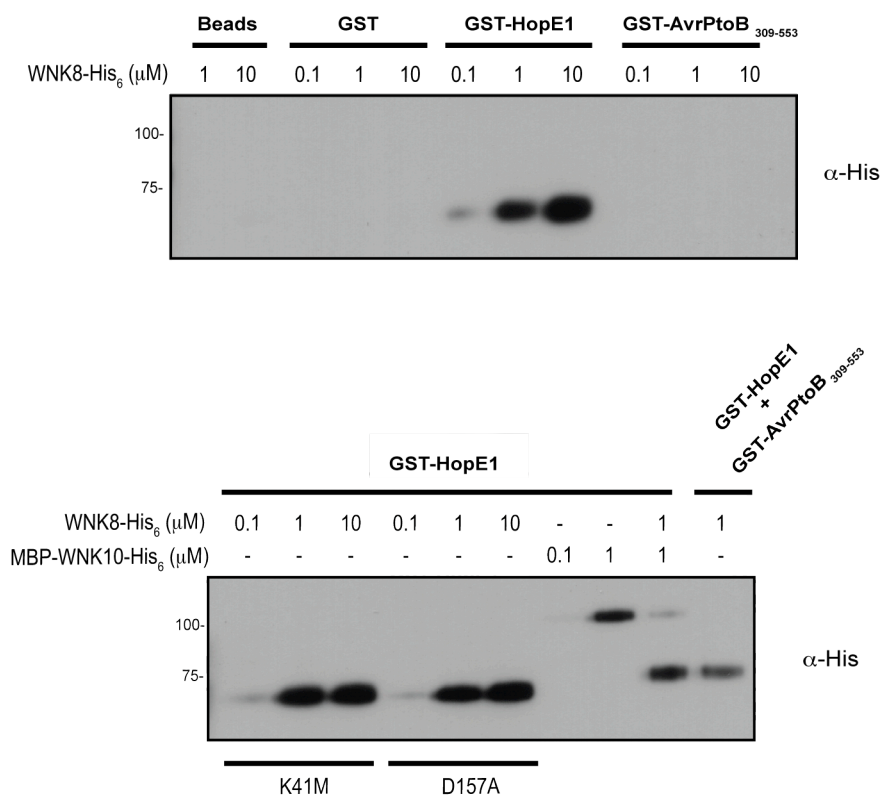
#### *HopE1 Can Suppress HopPsyA Induced HR in Nicotiana tabacum and Arabidopsis Ws-0*

It has been previously shown that HopE1 can suppress HR induced by the avirulence effector, HopPsyA. This effector is encoded on the pHIR11 cosmid from *Pseudomonas syringae* pv. *syringae* 61 and when the cosmid is delivered in the non-host pathogen *Pseudomonas fluorescens* it is able to cause HR in *N. tabacum* and *A. thaliana* Ws-0 (Jamir et al., 2004). We were able to successfully replicate the previously published results using the same non-host pathogen system to show that when *hopE1* is expressed in the vector pML123, it suppresses HopPsyA induced HR in both *N. tabacum* (Fig. 11A) and in *A. thaliana* Ws-0 (Fig. 11B). The experimental method as presented by

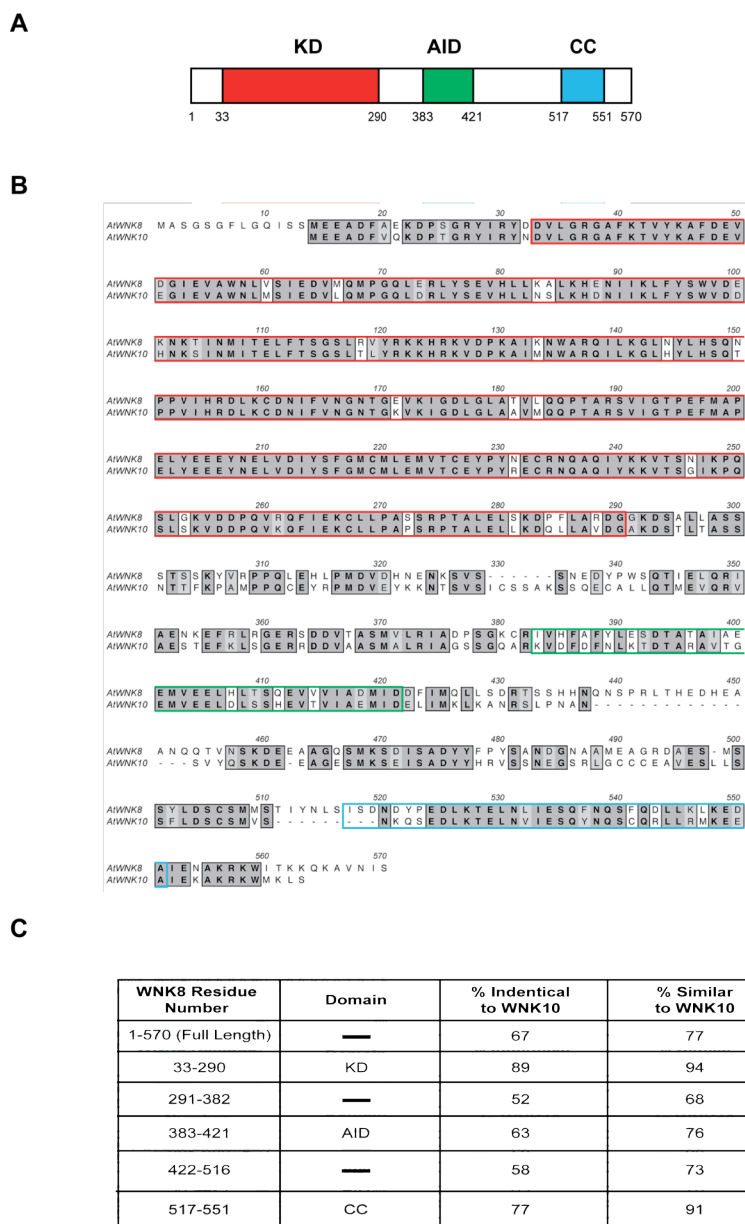
Jamir and colleagues is a valuable tool for future research since it allows a single effector to be tested for HR suppression activity. We are able to use this system to screen possible HopE1 mutants for loss of phenotype while avoiding experimental artifacts introduced when *Pst* WT is used. These findings are also important because in pathogenesis progression, the goal of the pathogen is not to kill the host early in infection, but use the host's resources to survive and expand. Understanding the mechanisms of how effectors like HopE1 are able to suppress the host HR are important because these effectors play a key role in the ability of bacterial pathogens to thwart host defenses long enough to cause systemic infection. Therefore, these effectors are important targets that can be used in developing novel strategies for combating bacterial phytopathogens.



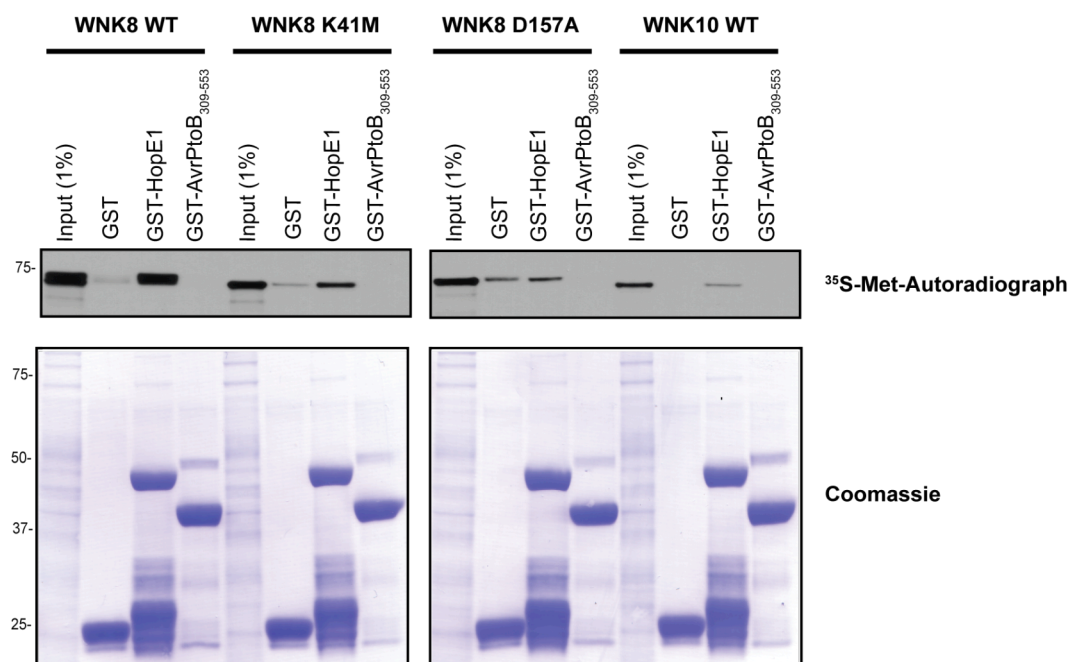
**FIGURE 1. Yeast two-hybrid reveals interaction of HopE1 and WNK8.** (A) L40 yeast expressing either pLEX-NA empty vector (EV) or pLEX-NA HopE1 were lysed, separated by SDS-PAGE and immunoblotted with an anti-LexA antibody to test for expression of the proper LexA fused bait protein. Letters after construct name refer to independent isolates. (B) Summary of sequencing results from positive L40 yeast clones selected for interaction of the bait (HopE1) and prey (*Arabidopsis* cDNA Library) by growth on SD-His-Trp-Ura media. (C) A forced yeast two-hybrid using L40 yeast cotransformed with prey and bait plasmids was performed. Three independent isolates for each prey protein were grown on SD-Trp-Ura media to test for yeast viability and on SD-Trp-Ura-His media to test for positive interactions.



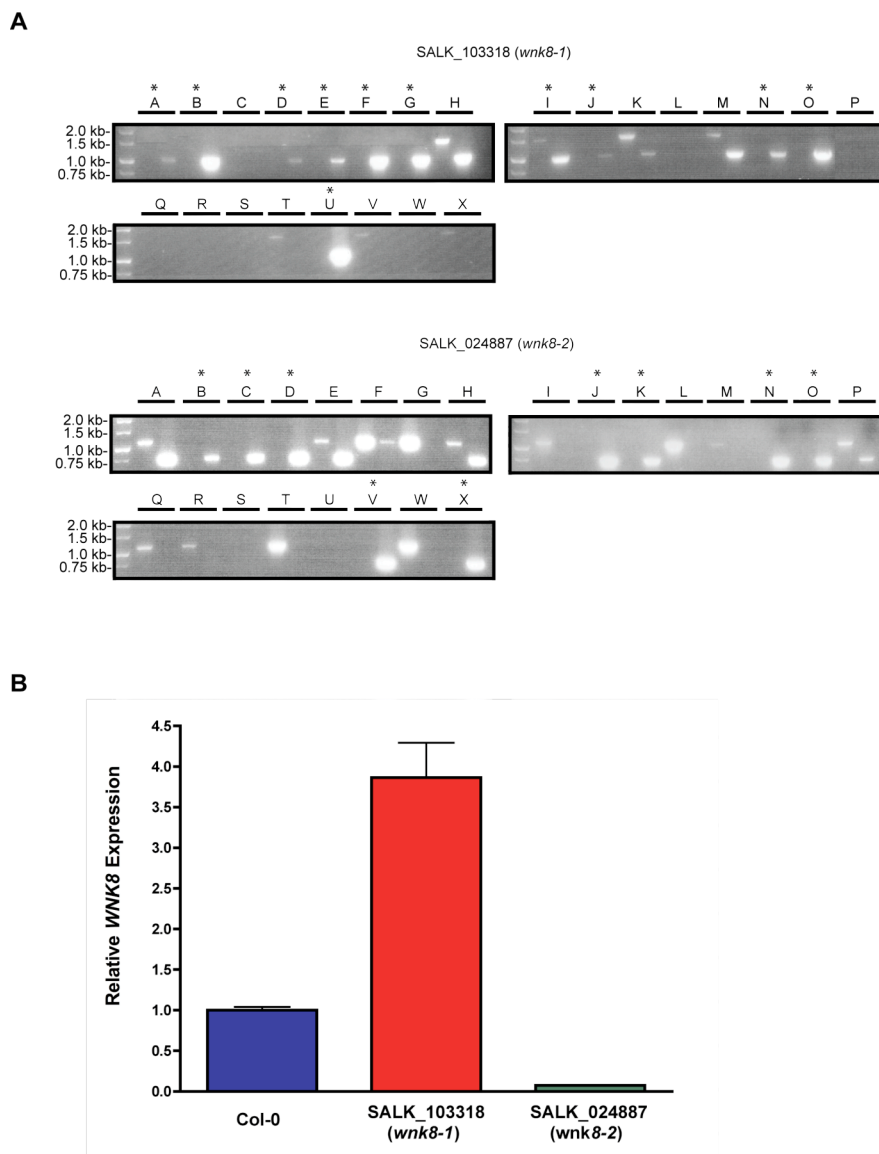
**FIGURE 2. Specific interaction of HopE1 and WNK8.** 1 μM GST, GST-HopE1 or GST-AvrPtoB 309-553 was added to either 0.1, 1 and 10 μM of WNK8 WT-His<sub>6</sub>, WNK8 K41M-His<sub>6</sub>, WNK8 D157A-His<sub>6</sub> or MBP-WNK10-His<sub>6</sub> and allowed to bind at 4°C. Glutathione agarose beads were then added to each sample, washed, eluted with 2x LDS-PAGE loading buffer, separated by SDS-PAGE and immunoblotted with an anti-His<sub>4</sub> antibody.



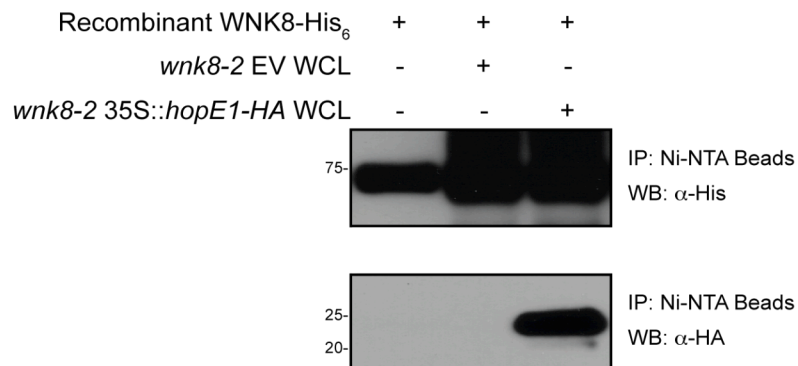
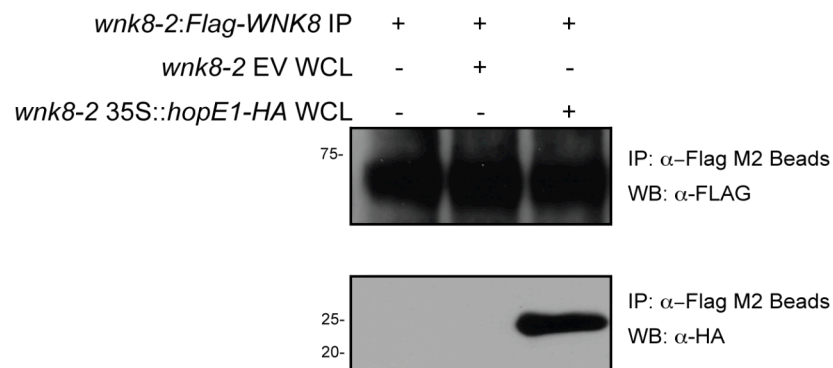
**FIGURE 3. Sequence analysis of WNK8 and WNK10.** (A) Schematic illustration of the domain structure of WNK8 and WNK10, which include a kinase domain (KD), an autoinhibitory domain (AID) and a predicted coiled-coil domain (CC). (B) ClustalW sequence alignment of full length WNK8 and WNK10. The kinase domain is outlined in red, the autoinhibitory domain is outlined in green and the coiled-coil domain is outlined in blue. (C) Comparison of WNK8 and WNK10 sequences in conserved domains and surrounding regions. Percent similarity is the percent of residues that either identical or chemically conserved.



**FIGURE 4. *In vitro* transcribed and translated WNK8 interacts with HopE1.** WNK8, WNK8 K41M, WNK8 D157A or WNK10 (all in pET21a) were *in vitro* transcribed and translated using a wheat germ extract TnT kit and 1% was removed following the reaction. GST, GST-HopE1 and GST-AvrPtoB 309-553 (20  $\mu$ g) was bound to glutathione agarose beads, washed, then added to the remaining TnT reaction, incubated at 4°C, washed, eluted with 2x LDS-PAGE loading buffer, separated by SDS-PAGE, transferred to a PVDF membrane and analyzed by autoradiography (top panel). Before autoradiography the PVDF membrane was coomassie stained for loading control (bottom panel).



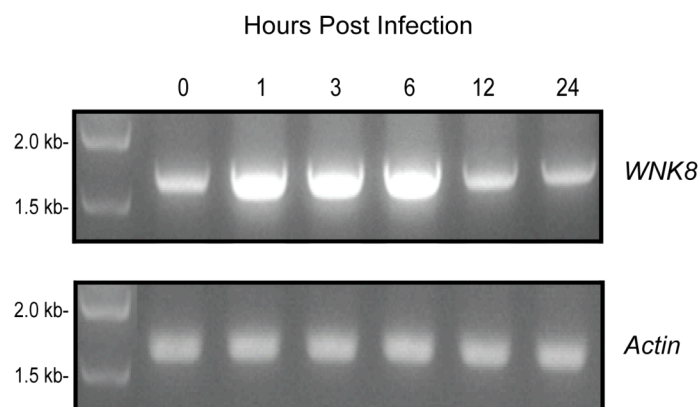
**FIGURE 5. Validating Salk *WNK8* T-DNA insertion lines.** (A) 24 independent plants (designated with the letters A-X) from each *WNK8* insertion line were tested for disruption of the *WNK8* coding sequence. One leaf from each plant was collected, genomic DNA isolated and PCR tested for homozygous insertion using two pairs of primers. The left lane of each sample represents the primer set consisting of a 5' and a 3' *WNK8* specific primer. If the insertion is present within the *WNK8* coding sequence no PCR product is expected. The right lane of each sample consists of an insertion specific primer and the 3' *WNK8* primer and if the insertion is present a band of 900 bp is expected in *WNK8-1* samples and 750 bp for *WNK8-2* samples. Homozygous lines are indicated by an asterisk (\*). (B) Homozygous T<sub>1</sub> Col-0, *WNK8-1* and *WNK8-2* plants were grown, RNA isolated, converted to cDNA and analyzed for relative *WNK8* expression (normalized to Col-0) by qPCR using *WNK8* specific primers.

**A****B**

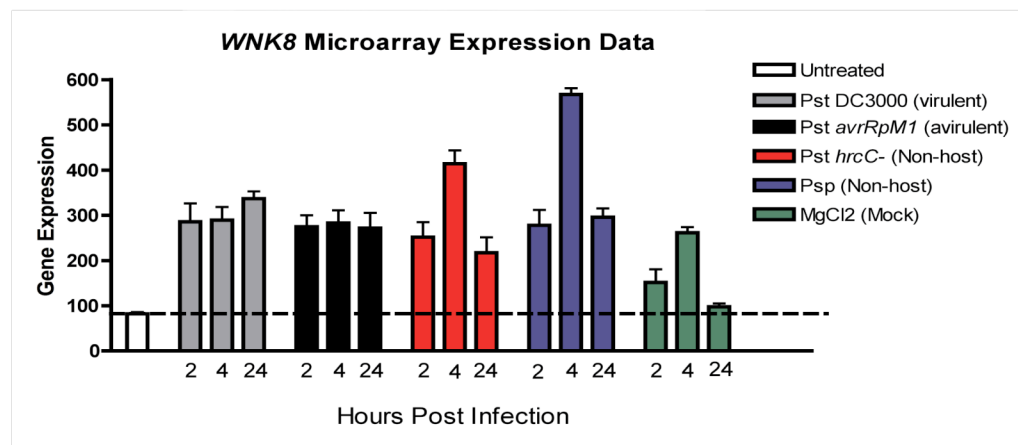
**FIGURE 6. HopE1 binds WNK8 in *Arabidopsis* cell extracts.** (A) *E. coli* purified WNK8-His<sub>6</sub> was bound to Ni-NTA agarose beads, washed, then WCL was added from either *wnk8-2* EV plants or *wnk8-2* 35S::*hopE1-HA* plants, allowed to bind, washed, eluted with 2x LDS-PAGE loading buffer, subjected SDS-PAGE and immunoblotted with anti-His<sub>4</sub> or anti-HA antibodies. (B) Flag-WNK8 was immunoprecipitated from *wnk8-2:Flag-WNK8* plant WCL using anti-FLAG M2 agarose beads, washed, then WCL was added from either *wnk8-2* EV plants or *wnk8-2* 35S::*hopE1-HA* plants, allowed to bind, washed, eluted with 2x LDS-PAGE loading buffer, separated by SDS-PAGE and immunoblotted with anti-Flag or anti-HA antibodies.



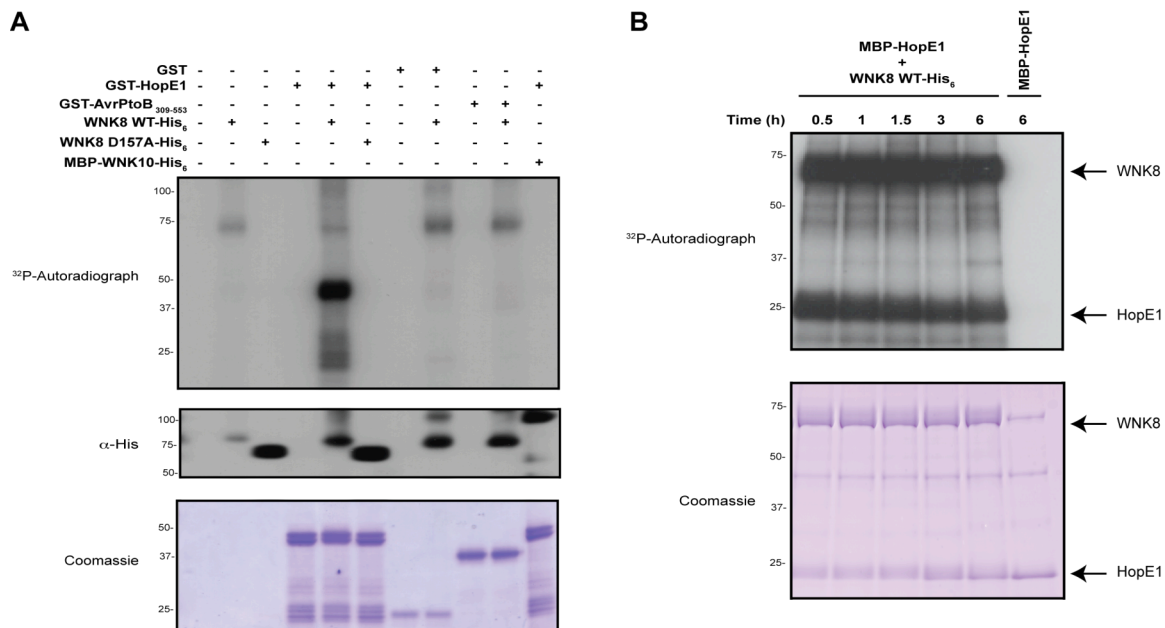
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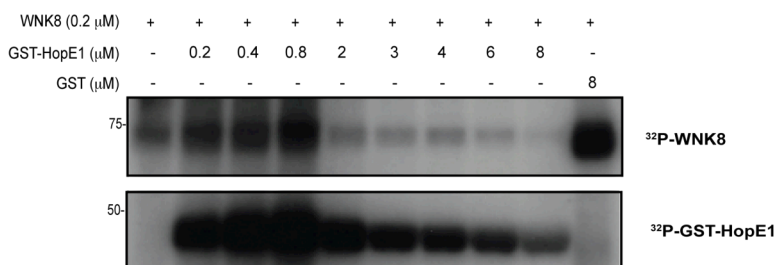


**FIGURE 7. *WNK8* expression is induced upon *Pst* infection.** (A) *Arabidopsis thaliana* Col-0 plants were infected with *Pst* and leaf tissue was collected at the indicated time points. RNA was then isolated from infected tissue, converted to cDNA using RT, PCR amplified using *WNK8* specific primers and analyzed by agarose gel electrophoresis. (B) Analysis of the gene At5g41990 (*WNK8*) using available microarray data of *A. thaliana* infected with multiple strains of *Pst*. Dotted line indicates baseline (untreated) *WNK8* expression.

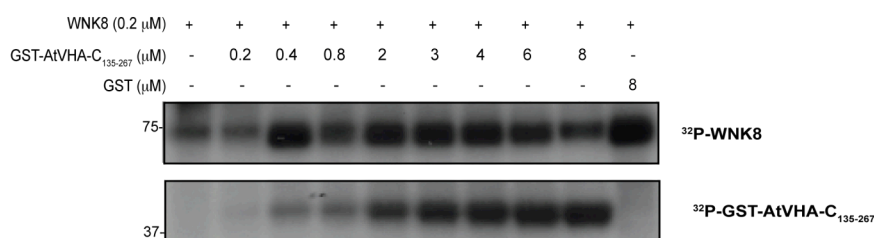


**FIGURE 8. HopE1 is phosphorylated by WNK8.** (A) 1  $\mu$ M HopE1 or appropriate control was combined with 0.25  $\mu$ M kinase (WNK8 WT, D157A, or WNK10) in the presence of  $\gamma$ - $^{32}$ P-ATP and necessary co-factors. Reactions were quenched with 4x LDS-PAGE loading buffer after 30 min and separated by SDS-PAGE for analysis. One-quarter of the total reaction volume was analyzed by autoradiography for phosphotransfer (top panel). One-quarter of the reaction was transferred to PVDF membrane and analyzed by immunoblotting with an anti-His<sub>4</sub> antibody for loading control of the kinases (middle panel). One-quarter of the reaction was analyzed by coomassie staining to show loading of substrates (bottom panel). (B) Kinase reactions were set up as in (A), but using MBP-HopE1. Reactions were then treated with thrombin at indicated time points to precipitate HopE1, washed twice with H<sub>2</sub>O, resuspended with 20  $\mu$ l 2x LDS-PAGE loading buffer, subjected to SDS-PAGE and analyzed by autoradiography (top panel). Coomassie stained gel of thrombin cleaved precipitate (bottom panel).

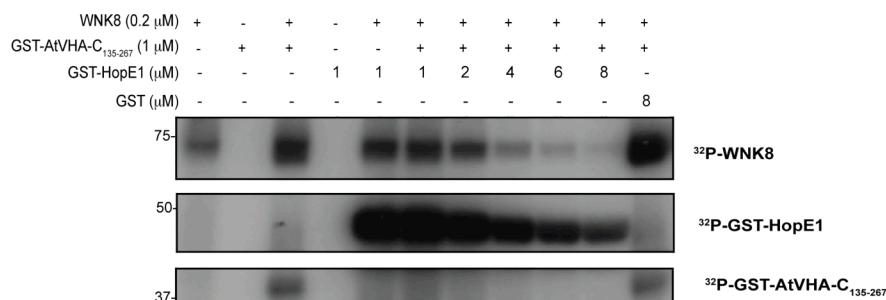
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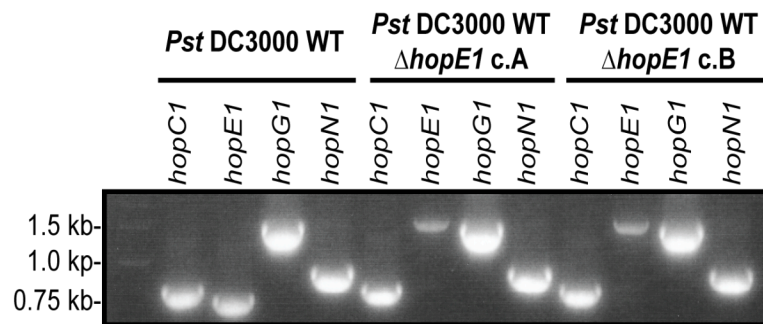
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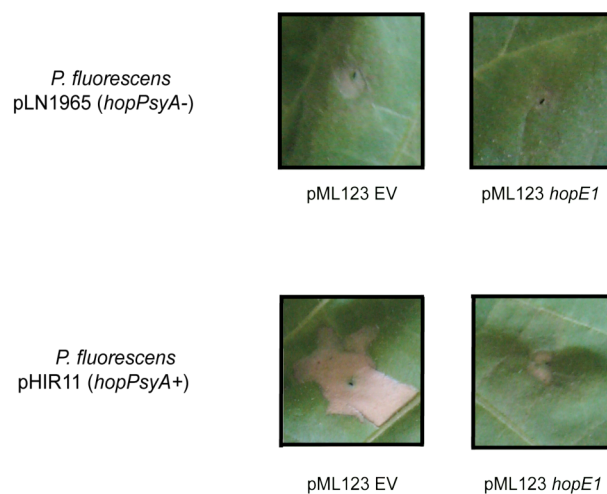


**FIGURE 9. HopE1 can inhibit the kinase activity of WNK8.** (A) Indicated amounts of WNK8 and GST were added with increasing amounts of GST-HopE1 in a standard kinase reaction for 30 min, quenched with 4x LDS-PAGE loading buffer, separated by SDS-PAGE and analyzed by autoradiography for inhibition of WNK8 autophosphorylation. (B) Kinase reactions were carried out as in (A), but increasing amounts of GST-AtVHA-C 135-267 were added instead of GST-HopE1. (C) Increasing amounts of GST-HopE1 were added to both WNK8 and AtVHA-C 135-267 and carried out as in (A).



**FIGURE 10. Creation of a *hopE1* knockout *Pst* DC3000 strain.** After plating on selective media, positive isolates (c.A and c.B) were PCR screened for *HopE1* and three other unique *Pst* DC3000 genes (*HopG1*, *HopC1* and *HopN1*) then separated by agarose gel electrophoresis. The presence of the resistance cassette was detected by a *HopE1* PCR product shift from 636 bp in length to 1500 bp (length of *hopE1* plus the length of the resistance cassette).

A



B

Construct	<i>P. fluorescens</i> strain	Fraction of leaves showing HR
Mock	—	0/10
pML123 EV	pLN1965 ( <i>hopPsyA</i> -)	1/15
pML123 <i>hopE1</i>	pLN1965 ( <i>hopPsyA</i> -)	2/15
pML123 EV	pHIR11 ( <i>hopPsyA</i> +)	12/15
pML123 <i>hopE1</i>	pHIR11 ( <i>hopPsyA</i> +)	4/15
pML123 <i>hopE1</i> -3xHA	pHIR11 ( <i>hopPsyA</i> +)	3/15

**FIGURE 11. HopE1 suppresses HopPsyA induced HR in both Tobacco and *Arabidopsis*.** (A) *N. tabacum* c.v. *Xanthi* leaves were infected with non-host pathogen *Pseudomonas fluorescens* strains either containing *hopPsyA* (pHIR11) or lacking *hopPsyA* (pLN1965). These strains also carried plasmids either expressing empty vector (EV) or *HopE1* to test for suppression of HR. (B) The same strains described in (A) plus a *Pf*(pHIR11 *HopE1*-3xHA) were used to infect *Arabidopsis* Ws-0 leaves which were then scored based on the presence or absence of HR response.

## Conclusion

Bacterial phytopathogens represent a persistent problem and cause millions of dollars of damage per year in the food industry. In recent years, research in this area has increased as the growing population begins to dwindle the food supply. One interesting observation is the ability of phytopathogens to continually evolve and develop new and better weapons to subvert host defense responses and continue to cause disease. In order to promote disease in the plant, pathogens inject an arsenal of effector proteins into the host cell through the use of a type III secretion system. These type III effector proteins employ a diverse set of molecular strategies, which allow the pathogen to continue to grow by suppressing the host defense response. These strategies often interfere with host signaling components, however only a few of these mechanisms are well defined and many others remain unknown. Here, we examined the poorly characterized *Pseudomonas syringae* pv. *tomato* DC3000 type III effector, HopE1, using multiple biochemical approaches that will direct future research in elucidating how HopE1 suppresses HR in *Arabidopsis*.

Our first step in dissecting the function of HopE1 was to find potential host target(s) through which the activity of HopE1 could be modulated. We performed a yeast two-hybrid screen using HopE1 and an *Arabidopsis* c-DNA library in order to identify proteins that potentially interact with HopE1. One of the interesting proteins identified in the yeast two-hybrid screen was the *Arabidopsis* serine/threonine protein kinase, WNK8. We show that HopE1 specifically binds WNK8 using purified proteins, wheat germ *in*

*in vitro* transcribed and translated proteins, as well as *Arabidopsis* cell extracts. We show that *WNK8* expression is induced upon *Pst* infection using RT-PCR analysis. There is a sizable increase in *WNK8* gene induction that occurs early in infection suggesting that *WNK8* may be involved in early *Arabidopsis* defense response. Future experiments would focus on characterizing the function of *WNK8* in early defense response. We also investigated whether HopE1 can be phosphorylated by *WNK8*. We clearly show that *WNK8* phosphorylates HopE1 using an *in vitro* kinase assay. To further study *WNK8* kinase activity, we examined if HopE1 can affect on the ability of *WNK8* to autophosphorylate or phosphorylate its only known endogenous substrate (AtVHA-C). Our data shows that HopE1 inhibits the autophosphorylation of *WNK8* as well as suppress *WNK8* phosphorylation of AtVHA-C.

Induction of HR is an important part of the defense response utilized by plants to protect themselves from invading bacteria. Although plants and other species have developed this generalized anti-microbial response, pathogens are still able to cause infection in susceptible hosts despite these responses. One example is the bacterial phytopathogen, *Pseudomonas syringae* pv. *tomato* DC3000, which causes bacterial speck disease in susceptible plants. This pathogen uses the TTSS to inject effector proteins into the host in order to carry out its virulence. Effectors such as HopE1 are able to suppress the host HR, which allows the bacteria to continue to grow and cause infection. However, HopE1 and many other effectors do not belong to a family whose biological activity is known. Understanding the activity of HopE1 is important because other type three effectors able to suppress the host HR may use a similar mechanism, but target different substrates. By understanding this mechanism, it is possible to identify a larger family of

these proteins through the use of bioinformatics. The larger family of functional equivalents could span many different pathogens that infect a broad range of hosts including humans, therefore could be used in developing novel strategies to combat these pathogens. Likewise, understanding the target(s) of HopE1 is important because these could also be conserved from species to species. These targets can be used to develop strategies to block modifications by the pathogen and inhibit its activity. Understanding how type three effectors can inhibit the plant HR will provide insight into the mechanisms employed by bacterial pathogens to subvert host defense responses.

Fully understanding *Pst* virulence will be an important step in reducing the number of crops lost to pathogen that cost manufactures and consumers billions of dollars per year. Also it has been estimated that by the year 2050, the world population will be greater than 9 billion. At this current rate of growth, the world's food supply is insufficient to feed the population; therefore steps to increase and protect our food supply must occur now in order to prepare for the future. Characterizing important proteins involved in the plant-phytopathogen relationship will help further progress our studies on ways to prevent pathogenesis in plants. The ultimate goal will be to capitalize on these findings to engineer pathogen resistant plants and crops. This type of approach has already been proven to work when researchers were able to produce *Pseudomonas* resistant tomato plants by introduction of the Pto gene in tomatoes during the 1970's and is still used today. Besides less crops being lost to disease, this research has lead to reducing the use and abuse of harmful pesticides in farming. Unlike with pesticides and antibiotics, pathogens have not been able become resistant to genetically modified crops even after over 30 years of use as in the case of Pto tomato plants. Using biochemical



approaches, we have further characterized the *Pseudomonas syringae* pv. *tomato* DC3000 type III effector, HopE1, and identified an *in vitro* target. Further experimentation will determine if this interaction occurs *in planta* and whether this has implications in the ability of HopE1 to suppress plant HR. This unique activity of HopE1 makes it a potential candidate in creating *Pseudomonas syringae* pv. *tomato* resistant plants and crops in the future.

## Materials and Methods

### *Yeast-Two Hybrid Assays*

The yeast strain L40 (MATa his3 $\Delta$ 200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)<sub>4</sub>-HIS3 URA3::(lexAop)<sub>8</sub>-lacZ GAL4) was used to test the interaction between HopE1 and a yeast two-hybrid *Arabidopsis* c-DNA library (Kim et al., 1997). Full length HopE1 was PCR amplified from *Pseudomonas syringae* pv. *tomato* DC3000 genomic DNA using standard PCR conditions and cloned in frame with LexA into the EcoRI/BamHI sites of the yeast vector pLEX-NA. To ensure proper expression of the LexA DNA binding domain fused bait protein, L40 yeast expressing either pLEX-NA EV or pLEX-NA HopE1 were lysed using standard protocol and resuspended in loading buffer. Samples were then separated by SDS-PAGE and immunoblotted with a rabbit anti-LexA antibody (Invitrogen). Yeast cells were transformed with bait and tested for autoactivation and then transferred with prey library according to standard protocols. Following transformation of the prepared bait/prey strain yeast two-hybrid positives were then tested for interaction by growth selection on SD-Trp-Ura-His minimal media plates. Positives were then grown in SD-Trp minimal media to drop out the bait plasmid and the library plasmid was isolated using a standard yeast plasmid isolation protocol. The resulting plasmid was then transformed into the DH5 $\alpha$  *E. coli* strain for amplification, isolated by plasmid miniprep kit (Invitrogen) and sequenced using pACT2 specific primers (5'-GGAATCACTACAGGGATG and 5'-CGATGCACAGTTG AAGTG). Resulting sequences were compared to the *Arabidopsis thaliana* using BLASTN to

identify the yeast two-hybrid positive clones. In the forced yeast-two hybrid experiment, full length WNK8 and WNK10 were PCR amplified from an *Arabidopsis* c-DNA library and cloned into the BamHI/SalI sites of pGADGH. The WNK8 K41M mutant was prepared by QuikChange Site-Directed Mutagenesis kit (Stratagene) using the manufacturer's instructions and WNK8 WT as a template, and was then cloned into pGADGH. L40 yeast were then cotransformed with bait and prey plasmids, selected on SD-Trp-Ura minimal media and then tested for interaction by growth on SD-Trp-Ura-His minimal media plates.

#### *Constructs, Proteins and Protein Purification*

Full length HopE1, AvrPtoB 309-553 and AtVHA-C 135-267 were cloned into the pGEX-KG vector, which contains a N-terminal GST tag, using standard PCR conditions. HopE1 and AvrPtoB were cloned from *Pst.* DC3000 genomic DNA and AtVHA-C was cloned from an *Arabidopsis* c-DNA library. Full length WNK8 WT was amplified from an *Arabidopsis* c-DNA library and cloned into the pET21a vector containing a C-terminal His<sub>6</sub> tag (Novagen). WNK8 K41M and WNK8 D157A mutants were produced by QuikChange Site-Directed Mutagenesis (Stratagene) using the manufacturer's instructions and pET21a-WNK8 WT as the template. MBP-WNK10-His<sub>6</sub> was created by cloning WNK10 amplified from an *Arabidopsis* c-DNA library into the pET28-MBP vector (N-terminal MBP tag and C-terminal His<sub>6</sub> tag, (Lee et al., 2008)). MBP-HopE1 was created by cloning PCR amplified HopE1 into the pET15b vector (Novagen). All proteins were expressed by transforming the appropriate vector into *E.*

*coli* BL21(DE3)RILP cells. Recombinant GST-HopE1 was purified by growing the strain in LB media at 37°C to an OD<sub>600</sub> of 0.7, then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 hrs at 12°C before harvesting the cells at 4°C. Due to the fact that GST-HopE1 co-purifies with the common *E. coli* chaperone GroEL, protein purification had to be carried out as previously described (Rohman and Harrison-Lavoie, 2000) in order to remove the GroEL except a column purification was performed using a 2 mL bed volume of GST•Bind Resin (Novagen) and eluted with 50 mM Tris-HCl pH=7.4, 0.15 M NaCl, 20 mM reduced Glutathione, 2.5% glycerol and 0.5 mM tris(2-carboxyethyl)phosphine (TCEP). All other proteins were purified by growing the appropriate strain in LB media at 37°C to an OD<sub>600</sub> of 0.7, then induced with 0.4 mM IPTG for 12 hrs at 23°C before harvesting the cells at 4°C. All His<sub>6</sub> tagged proteins except MBP-WNK10-His<sub>6</sub> were purified by affinity chromatography as previously described (Mitchell and Marletta, 2005) using Ni-NTA superflow agarose resin (Qiagen). All maltose binding protein (MBP) tagged proteins (including MBP-WNK10-His<sub>6</sub>) were purified as previously described (Lee et al., 2008) by affinity chromatography using amylose resin (New England Biolabs). After purification all proteins were concentrated using Amicon Ultra concentrators (Millipore) and buffer exchanged into protein storage buffer (50 mM HEPES pH=7.5, 50 mM NaCl, 2.5% glycerol and 0.5 mM TCEP).

#### *Recombinant Protein Binding Assays*

Indicated molar amounts of each protein were added together and the reaction volume was taken up to 100 µl with binding buffer (10 mM Tris pH=7.4, 150 mM NaCl

and 0.5% (v/v) Triton T<sub>x</sub>-100). Proteins were rotated for 2 hrs at 4°C, then 40 µl of pre-equilibrated (washed 3x with binding buffer) GST•Bind Resin (Novagen) slurry was added to each reaction, incubated for 2 hrs at 4°C, washed 6x (3x brief and 3x 5 min) with 400 µl binding buffer, and eluted with 40 µl 2x LDS-PAGE loading buffer. Samples were then heated at 95°C for 10 min and 5 µl was loaded for SDS-PAGE and immunoblotted with an anti-His<sub>4</sub> antibody (Qiagen).

#### *In vitro Transcription and Translation (TnT) Binding Experiments*

WNK8 WT, WNK8 K41M, WNK8 D157A and WNK10 WT were all cloned into pET21a for *in vitro* expression under the control of a T7 RNA polymerase promoter present in the vector. The kinases in pET21a were *in vitro* transcribed and translated in wheat germ extract (Promega) in the presence of <sup>35</sup>S-methionine (PerkinElmer) according to manufacturer's instructions. Transcription and translation reactions were carried out at 30°C for 2 hr and after the reaction 1% of the input (0.5 µl) was removed and added to 9.5 µl 2x LDS-PAGE loading buffer. Recombinant GST, GST-HopE1 or GST-AvrPtoB 309-553 proteins (20 µg each) and 200 µl binding buffer (10 mM Tris pH=7.4, 150 mM NaCl and 0.5% (v/v) Triton T<sub>x</sub>-100) was added to 80 µl of pre-equilibrated (washed 3x with binding buffer) GST•Bind Resin (Novagen) slurry and rotated at 4°C for 2 hours. The bound proteins were then washed and the TnT reaction mixture was then added to give a final reaction volume of 250 µl and rotated for 4 hrs at 4°C. Reactions were washed 4x with binding buffer, eluted with 40 µl 2x LDS-PAGE

loading buffer and 20  $\mu$ l was used for SDS-PAGE. The gels were then transferred to a PVDF membrane then stained with 0.1% Coomassie Blue R (Sigma) in 50% methanol, destained with 50% methanol/10% acetic acid (2-3 washes), washed twice with water, and air-dried. The dried membrane was then analyzed by autoradiography using a low energy isotope-intensifying screen (Kodak BioMax) for 8 hr at  $-80^{\circ}\text{C}$ .

#### *RT-PCR Analysis of AtWNK8 Expression*

*Arabidopsis thaliana* Col-0 plants were grown in a Promix-HP:vermiculite (2:1) soil mix under a 9 hr photoperiod at  $22^{\circ}\text{C}$ . Leaf tissue was collected at indicated time points from 4-6 week old plants infected by syringe infiltration of *Pst* DC3000 ( $\sim 3.75 \times 10^7$  cfu  $\text{ml}^{-1}$ ) as previously described (Shao et al., 2003). RNA was isolated using the RNeasy plant mini kit (Qiagen) and cDNA was generated from total RNA (1  $\mu$ g) using the SuperScript III kit (Invitrogen) and oligo(dT) primers according to manufacturer's instructions.. cDNA (1  $\mu$ g) was then amplified using standard PCR with *WNK8* and *actin* specific primers (*WNK8*: 5'-AAAACATATGGCTTCTGGTTCTGGATTTT TAGGTCAGATATCG and 5'-AAAAGCGGCCGCAGAGATGT TAACTGCTTTT TGCTTTTTTCGTAATCC *actin*: 5'-ATGGCAGACGGTGAGGATATTCA and 5'-GCC TTTGCAATCCACATCTGTTG) and using Master Taq (Eppendorf). Reactions were analyzed by agarose gel electrophoresis.

*In vitro Kinase Assays*

Indicated amounts of the kinases and other recombinant proteins were added in a total reaction volume of 60  $\mu\text{l}$  in kinase reaction buffer (25 mM Tris-HCl pH=7.0, 0.2 mM EDTA, 5 mM  $\text{MnCl}_2$ , 25  $\mu\text{M}$  ATP and 5  $\mu\text{Ci}$   $\gamma$ - $^{32}\text{P}$ -ATP (PerkinElmer)). Reactions were incubated at 30°C for 30 min and quenched by the addition of 20  $\mu\text{l}$  4x LDS-PAGE loading buffer and incubation at 95°C for 10 min. Samples (20  $\mu\text{l}$  each) were subjected to SDS-PAGE on triplicate gels used for analysis. One gel was transferred to PVDF membrane and immunoblotted with anti-His<sub>4</sub> antibody (Qiagen) to detect the His<sub>6</sub> tagged kinases as a loading control. The second gel was analyzed by coomassie staining as a loading control for all other proteins and the third gel was dried down under vacuum and analyzed by autoradiography for  $^{32}\text{P}$  incorporation. For time course kinase assay experiments, 10.2  $\mu\text{M}$  of thrombin cleavable MBP-HopE1 was added to 2.75  $\mu\text{M}$  WNK8-His<sub>6</sub> and kinase reaction buffer in a total volume of 30  $\mu\text{l}$ . At indicated time points 5  $\mu\text{l}$  of 10X thrombin cleavage buffer (Sigma) and 1  $\mu\text{l}$  of thrombin (Gift from Betsy Komives) were added and incubated for 1 hr at 30°C to precipitate cleaved full length HopE1. Precipitate was then spun down for 3 min at 16,100 x g, the supernatant was removed, washed 2x with ddH<sub>2</sub>O, and the pellet was resuspended in 20  $\mu\text{l}$  2x LDS-PAGE loading buffer. The entire reaction was subjected to SDS-PAGE, coomassie stained, dried under vacuum and then analyzed by autoradiography. For kinase inhibition studies, indicated amounts of each protein were added in a standard kinase assay.

Aliquots (20  $\mu$ l) of the total reaction was separated by SDS-PAGE and analyzed by autoradiography.

#### *AtWNK8 T-DNA Insertion Line Analysis*

Both *WNK8* insertion lines, *wnk8-1* (SALK\_103318) and *wnk8-2* (SALK\_024887) were identified using the SIGNAL Salk website. Leaves from adult plants were collected, the genomic DNA was isolated by standard procedures, and genotyping PCR was performed. PCR analysis was carried out with 500 ng of genomic DNA and the Master Taq Kit (Eppendorf) using primers and methods according to the SIGNAL Salk T-DNA primer design guidelines (<http://signal.salk.edu/tdnaprimers.2.html>). *wnk8-1* primers used were as follows; left primer (LP) 5'-AAAGATCCTTCTGGCCGTTAC and right primer (RP) 5'-TGCCATGAATTCAGGAGTACC. *wnk8-2* primers used were: LP 5'-CAGCAGATCTTGGAAG GACTG and RP 5'TACTCCTGAATTCATGGCACC. The insertion specific primer used for both lines was LBb1 5'-GCGTGGACCGCTTGC TGCAACT. For RT-qPCR analysis, an adult leaf from homozygous insertion mutants was collected, the RNA was isolated using the RNeasy plant mini kit (Qiagen) and cDNA was generated from total RNA (1  $\mu$ g) using the SuperScript III kit (Invitrogen) and oligo(dT) primers according to manufacturer's instructions. RT-qPCR reactions were run on a MX4000 Multiplex QPCR machine (Stratagene) using Power SYBR Green PCR Mastermix kit (Applied Biosystems). Primer pairs for *WNK8* (target) were 5'- ATTGC TGACCCGTCTGGTA and 5'- GCGATTGCTGTTGCTGTG and for *TUA3* (endogenous control) 5'-GTATT GAACGCATCGTGTG and 5'-TGGGAGCTTTACTGTCTCGAA.



All amplification efficiencies were verified for 100% efficiency before use. Ct values were generated using default parameters and relative expression values were calculated using the formula  $2^{-((Ct_{WNK8\ wnk8-1\ or\ wnk8-2} - Ct_{TUA3\ wnk8-1\ or\ wnk8-2}) - ((Ct_{WNK8\ Col-0} - Ct_{TUA3\ Col-0}))}$ . Data presented is the mean of the fold difference in *WNK8* transcript compared to Col-0 (no *WNK8* gene insertion) of 3 technical replicates. Error bars represent the standard deviation of the fold change among the 3 technical replicates.

#### *Pst DC3000 HopE1 Knockout Strain*

The *hopE1* knockout strain was created by using the pJQ200SK suicide vector gene replacement method as previously described (Badel et al., 2006; Quandt and Hynes, 1993). Briefly, 2500 bp upstream and downstream of *hopE1* in the *Pst* genome were cloned so that they flanked a kanamycin resistance cassette, which was used for selection of recombinants. The flanking sequence allows for double homologous recombination of the kanamycin cassette within the *hopE1* gene upon induction of a conditional lethal gene encoded by the suicide vector.

#### *HR Suppression Assays*

Suppression of HopPsyA induced HR by HopE1 was performed exactly as described (Jamir et al., 2004). Briefly, adult *N. tabacum* cv. *Xanthi* or *A. thaliana* Ws-0 leaves were infiltrated with *Pseudomonas fluorescens* stains (both gifts from James Alfano) carrying HopPsyA (pHIR11) or lacking HopPsyA (pLN1965) and contain

pML123 EV, pML123 *hopE1* or pML123 *hopE-3xHA*. The *P.f.* pLN1965 strain is identical to *P.f.* pLN18 (Jamir et al., 2004) but allows for use of the broad-host-range plasmids encoding kanamycin resistance. *HopE1* was cloned into over expression vector pML123 using 36 bp upstream and 26 bp downstream in order to include the RBS of HopE1. The pML123 plasmids were introduced into *Pseudomonas* strains (grown on KB media at 28°C) via triparental mating using a DH5 $\alpha$  helper strain carrying the plasmid pRK2013.

#### *Co-Binding Experiments in Arabidopsis Cell Extracts*

HopE1 tagged with a HA epitope upstream of the stop codon was cloned into the dexamethasone inducible binary vector pTA7002. The complete *WNK8* gene (coding sequence, 1000 bp upstream and 1000 bp downstream) along with an N-terminal flag epitope tag was cloned into the binary vector pJHA212K under the control of its native promoter. The resulting vectors were transformed into *WNK8-2* plants via *Agrobacterium tumefaciens* GV3101-mediated floral dipping (Clough and Bent, 1998). T<sub>1</sub> *wnk8-2* 35S::*hopE1-HA* plants were isolated by kanamycin selection on ½ MS-MES agarose plates and T<sub>1</sub> *wnk8-2:Flag-WNK8* plants were isolated by hygromycin selection on ½ MS-MES agarose plates. Positive plants were transferred to soil after selection, genotyped and the seed from positives were collected. For WNK8-His<sub>6</sub> binding experiments, 20  $\mu$ g recombinant WNK8-His<sub>6</sub> was added to IP buffer (20 mM Tris pH-7.5, 150 mM NaCl, 1% Triton T<sub>x</sub>-100 and 2x plant protease inhibitor cocktail (Sigma)) up to 100  $\mu$ l. Next, 40  $\mu$ l of pre-equilibrated (washed 3x with IP buffer) Ni-NTA

superflow resin (Qiagen) slurry was added to each reaction and rotated for 2 hr at 4°C. T<sub>2</sub> *wnk8-2 35S::hopE-HA* plants were induced with 30 μM DEX for 8.5 hrs before tissue was collected and homogenized using a mortar and pestle, resuspended in 5 μl/mg tissue with IP buffer, rotated at 4°C for 1 hr, spun at 10,000 x g for 5 min and the protein concentration in the supernatant was determined using Bradford Protein Assay Reagent (Bio-Rad). Lysate (4 mg) was pre-cleared for 1 hr at 4°C with 40 μl of pre-equilibrated (washed 3x with IP buffer) Protein A agarose slurry (Invitrogen) in a 800 μl reaction volume. 220 μl of washed (3x) WNK8-His<sub>6</sub> bound to Ni-NTA resin was added to 780 μl pre-cleared lysate, rotated for 6 hr at 4°C, washed 5x (2x 5 min), and eluted with 50 μl 2x LDS-PAGE loading buffer. Pull downs (20 μl) and WCL (50 μg) were subjected to SDS-PAGE in duplicate and immunoblotted with an anti-HA antibody (Roche) to detect HopE1-HA and an anti-His<sub>4</sub> antibody (Qiagen) to detect WNK8-His<sub>6</sub>. For Flag-WNK8 binding experiments, T<sub>2</sub> *wnk8-2:Flag-WNK8* plant tissue was homogenized using a mortar and pestle, resuspended in 5 μl/mg tissue with IP buffer, rotated at 4°C for 1 hr, spun at 10,000 x g for 5 min and the protein concentration in the supernatant was determined using Bradford Protein Assay Reagent (Bio-Rad). Lysate (4 mg) from both T<sub>2</sub> *wnk8-2:Flag-WNK8* and T<sub>2</sub> *wnk8-2 35S::hopE1-HA* was pre-cleared for 1 hr at 4°C with 40 μl of pre-equilibrated (washed 3x with IP buffer) Protein A agarose slurry (Invitrogen) in a 800 μl reaction volume. Next, 50 μl of pre-equilibrated (washed 3x with IP buffer) mouse anti-FLAG M2 agarose bead slurry (Sigma) was added to *wnk8-2:Flag-WNK8* pre-cleared lysate and rotated for 2.5 hr at 4°C. Washed (3x) Flag-WNK8 (220 μl) bound to anti-FLAG M2 agarose beads was added to 780 μl pre-cleared T<sub>2</sub> *wnk8-2 35S::hopE1-*

*HA* lysate, rotated for 6 hr at 4°C, washed 5x (2x 5 min), and eluted with 50 µl 2x LDS-PAGE loading buffer. Pull downs (20 µl) and WCL (50 µg) were subjected to SDS-PAGE in duplicate and immunoblotted with an anti-HA antibody (Roche) to detect HopE1-HA and a rabbit anti-FLAG antibody (Santa Cruz Biotechnology) to detect Flag-WNK8.

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